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RESEARCH ARTICLE

The Effect of Fluence on Neutrophil Kinetics in Zebrafish Wounds Using Real-time In Vivo Imaging

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ABSTRACT

Background: Neutrophils participate in a cooperative defense strategy with macrophages following tissue injury. Application of low dose electromagnetic radiation via 635 nanometers wavelength can enhance macrophage recruitment to the wound, decrease tissue levels of ROS and speed healing. The impact of this wavelength on neutrophil activity *in vivo* is not well-described. Given that wound healing can be enhanced if collateral damage by neutrophils within the wound is decreased, we investigated how neutrophil kinetics changed during these accelerated reparative responses.

Methods: Tg(mpx:dendra2) line zebrafish (Danio rerio), transgenic for the fluorescent neutrophil marker, *mpx-dendra2*, underwent defined fin tissue injury, and were randomized to one of two laser treatment groups, 3 J/cm^2 (n=57), or 18 J/cm^2 (n=69) or to the control group, 0 J/cm^2 (n=164). Electromagnetic wave exposures were administered by a 635 nanometers continuous 5mW Helium:Neon laser with recipients randomized by dose delivered. Fish were three-dimensionally time-lapse imaged 30-120 minutes post injury (mpi) and wound healing documented at 24 hours post-injury. Individual neutrophil movement was tracked according to distance from wound center.

Results: The lower treatment fluence, 3 J/cm^2, significantly decreased neutrophil migration speed into the wound, increased reverse migration, and promoted stasis outside the adjacent wound area when compared to control and higher energy doses. The 3 J/cm^2 treatment groups also exhibited more rapid wound closure when compared to control or higher fluence.

Conclusions: Unlike our previous work in macrophages in which low fluence treatment enhanced the speed of forward directed migration into the wound, the response of neutrophils was decreased speed into the wound, increased reverse migration, and stasis outside the areas of the wound edges. These findings advance the notion that low fluence treatments reduce neutrophil inflammatory responses within the wound by their reduced presence within the wound. Reduced neutrophil-mediated collateral damage may work in concert with enhanced macrophage wound activity to promote faster wound healing.

Abbreviations

- ROS: reactive oxygen species
- nm: nanometers
- J/cm^2: joules
- ATP: adenosine triphosphate
- μm: micrometers
- mg: milligrams
- mL: milliliters
- mW: milliwatt
- nm: nanometers
- PBMT: photobiomodulation

Introduction

Following tissue injury, neutrophils are the first inflammatory cells recruited to the wound. Upon arrival, they dominate the wound microenvironment, vigorously defending against infection by producing antimicrobial peptides and proteases to kill invading pathogens [1,2]. Armed with cell surface receptors which are triggered by markers of the inflammatory environment, neutrophils can also unleash a destructive torrent of reactive oxygen species, ROS, against wound pathogens[3] . While effective against invading pathogens, ROS production and associated weapons of defense come at the cost of the collateral damage sustained by surrounding tissues [4]. Control of neutrophil-related tissue damage is tightly regulated through the induction of neutrophil apoptosis. Tissue resident macrophages remove bacteria as well as foreign debris, dead cells, and importantly, signal for neutrophil apoptosis [5]. Neutrophil programmed cell death can be induced by the release of death receptor ligands, such as tumor necrosis factor-α (TNF-α) and Fas-ligand by wound macrophages [6]. Macrophages phagocytose apoptotic neutrophils transitioning the microenvironment away from inflammation and towards repair. Phagocytosis triggers macrophage production of anti-inflammatory cytokines which is associated with the transition of the inflammatory type I macrophage to the pro-regenerative type II macrophage[7]. Speeding the transition from an inflammatory to regenerative wound microenvironment is of great therapeutic interest and presents a present gap of knowledge in the field.

Energy for all cellular activity is driven by adenosine triphosphate (ATP). The delivery of electromagnetic waves can augment mitochondria production of ATP, by photobiomodulation therapy (PBMT) [8]. Increased mitochondrial metabolism and ATP synthesis has been proposed as the mechanism underpinning reduced inflammation [9] and enhanced wound healing [10] following treatment with light of these wavelengths [11]. In our previous study, we found that the delivery of 635nm light at a low fluence of 3J/cm^2 led to faster, directed macrophage migration to the wound and higher macrophage retention within the wound when compared to untreated wound macrophages [12]. This response was not observed when higher fluence of 18J/cm^2 was delivered, suggesting that the cellular utilization of low dose energy had a threshold above which additional energy could not further enhance function. Wounds treated with low dose fluence demonstrated 50 percent faster wound healing [14].

Because treated macrophages arrived at the wound faster and were retained for a longer period of time, we hypothesized that the increase in available mitochondrial energy fueled the macrophages to progress more efficiently through their ordinary responses, clearing tissue debris, apoptotic cells, and more rapidly transitioning to type II macrophages. This hypothesis has been supported by the known amounts of available energy used to maintain the differential metabolic states of Type I and Type II macrophages [13], with greater energy required for reparative responses.

In our previous study, we did not ascertain how the neutrophils were responding to additional available energy. Our motivation for this study was to use the framework of accelerated wound healing to measure the neutrophil kinetics and define their modified behaviors in response to augmented energy availability via photobiomodulation.

Materials and Methods

Preparation of Zebrafish

The Tg(mpx:dendra2) zebrafish (Danio rerio) line was purchased through the Zebrafish International Resource Center (Cat.#ZL6217), in which the promoter mpx was used to drive dendra2 expression in neutrophils (Figure 1A). Zebrafish larvae were maintained in 100 mm glass dishes at 28° C in E3 embryo media that was reconstituted in distilled water to a 1X solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) and supplemented with 0.05 % methylene blue . Larvae were anesthetized, as per American Veterinary Medical Association, AVMA guidelines, using buffered Tricaine (3-aminobenzoic acid ethyl ester), at certain time-points as described below (Sigma #A5040, 168 mg/L, adjusted to pH 7.0 using $NaHCO_3$). Zebrafish larvae were humanely euthanized following experimental procedures, first with 400 mg/mL Tricaine solution and then with 10 % sodium hypochlorite solution as per 2013 AVMA guidelines. Water quality, spawning, and housing was performed according to standard protocols. All animal procedures, conditions, and ethics described herein were approved by the Animal Care Committee at the University of Illinois at Chicago.

Wound Placement and Measures of Healing

Zebrafish were anesthetized (168 mg/L, Tricaine), immobilized (1% low melting point agarose solution), and underwent single caudal fin incisions using a 28-gauge sterile needle. The injury was approximately 50 µm to 100 µm from the notochord (Figure 1B). Animals that did not meet uniformity in wound perimeter size (320 ± 50 µm), distance from notochord, or demonstrated small breaks or nicks in the outline of the embryonic fin fold were euthanized and not included in the rest of the study. Wound healing was measured using Image J 24 hours after injury, measuring the percent reduction of each side of the V-shaped wound as previously reported[14].

Figure 1

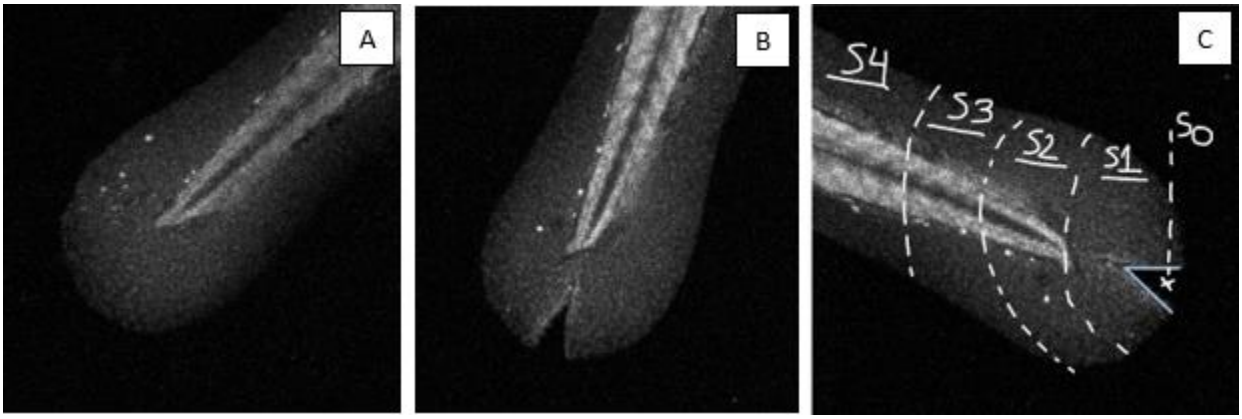

Figure 1*. Confocal images were taken of zebrafish 3 days post fertilization before (A) and after (B) tail injury. Image C shows the four areas localizing neutrophil movement reference to the wound center (s0): section one (s1), 150 micrometers encompassing the wound, from s0 to the notochord, section two (s2), 150 micrometers adjacent to s1, section three (s3), 150-300 micrometers from s1, section four(s4), 300-450 micrometers from s1. Wound region lines were curved for exact same distance from the wound center.*

Laser Irradiation for Time-lapse Imaging

Photobiomodulation was administered approximately 30 minutes post injury through a research grade 5mW (laserPower Source box: Zerona Medical Laser, Model #: EML-2245; Erchonia Corporation, McKinney, Tx) emitting a 635 nm continuous wave. Laser spot was elliptical at 0.25 cm 2 (4 mm x 2 mm) at the delivery site. Laser dosing was quantified in energy fluency (Joules/cm 2). Animals were randomly assigned to 3 treatment groups: 0 J/cm^2 (n=27 fish, n= 163 cells), 3 J/cm^2 (n=11 fish, n= 70 cells), and 18 J/cm^2 (n=8 fish, n=58 cells). Upon completion of photobiomodulation, all larvae were alive. Cells were localized according to distance from wound center and were tracked for 120 minutes post treatment with time intervals analyzed as follows: 30-60 mins, 30-90 minutes, and 30-120 minutes.

Time-lapse Imaging

All time-lapse images were acquired using ZEN® software with a Zeiss LSM 510 confocal Microscope with an NA 0.75/10X objective to image the zebrafish in time-lapse conditions. Two fluorescent channels were filtered, via ZEN® software, with the following excitations: 488 nm at 0.80 mW and 561 nm at 0.08 mW for dendra2 respectively. Three-dimensional time-lapse images were taken in 60 to 75-second intervals from 30 to 120 minutes post injury. The lateral pixel resolution was 1.64 µm and the image dimensions were 512 x 512 pixels. Using microscope programmed automation, up to 8 fish were imaged per session with at least one non-wounded fish for group baseline comparative analysis.

Analysis of Time-lapse Images

All time-lapse images were analyzed granularly using open source Zirmi software in MATLAB R2015a[14]. Quantification of cell kinetics was based on the centroid positions of the injury[14]. Neutrophil centroid positions were tracked using PhagoSight software's keyhole algorithm and visualization tools for proofing[12]. To analyze the extracted data granularly, wound regions were normalized, orienting positions with respect to the centroid or Position Zero (s 0) of the wound margin for discrete distance to wound determinations[14]. Zirmi software was used to manually trace wound region approximately 65 μm radially extended from the wound margin. Zirmi software was also used to select neutrophil tracks that matched a selection criterion or of distinguishable centroid positions in over 70 % of all frames and categorized as static when movement did not exceed 0.9 μm. Error-prone tracks of neutrophil near wound region were eliminated from analysis if they were aggregated together. Neutrophil migration was analyzed (30 to 120) minutes post-injury. Time intervals were defined as T1 [30 to 60 minutes post-injury], T2 [30 to 90 minutes post-injury], and, T3 [30 to 120 minutes post-injury].

Statistical Analysis

Statistical Analyses were performed in GraphPad Prism and Microsoft Excel. Linear correlations were performed to derive Pearson r coefficients. P values were used for testing the hypothesis that there is no relationship between the observed phenomenon (null hypothesis). P values of 0.05 were considered significant.

Results

Wound size was reduced by 50% as was reported previously [14]. We localized neutrophil movement data following injury over time and space using Image J as previously described for macrophages by Paredes, et al, [14]. Wound areas are shown in Figure 1c.

Neutrophil activity demonstrated significant differences across treatment groups in spatial domain 2, S2 [Figure 1c]. In this section, the neutrophils treated with 3 J/cm^2 exhibited the greatest reduction in forward wound migration and the greatest amount of stasis when compared to naïve neutrophils or those treated with 18 J/cm^2 [Figures 2b, 2c]. This ultra-low dose energy treatment appeared to induce a hesitancy of neutrophils entering the wound. Treated neutrophils did not seem to travel directly to the wound edge as frequently as naïve controls but instead chose to stay in the zone just outside the wound which was marked as S2 [Figure 1c].

Review of the first 120 minutes after injury demonstrated several significant differences between treatment groups. The geometric mean velocity observed in the 3 J/cm^2 treatment group, 4.30 ± 0.22 μm/min, was significantly decreased compared to control, 4.78 ± 0.12 μm/min, ($p < 0.01$) and the 18 J/cm^2 treatment, 5.05 ± 0.23 μm/min, ($p<0.01$), [Figure 2a]. Using the Forward Migration Index, a measure of discrete

movements toward the wound, we observed that treated groups demonstrated less movement toward the wound than control with 3 J/cm^2 demonstrating significantly slower migration to the wound, 0.59 ± 0.06 vs 0.71 ± 0.02 (p=0.03). There was no difference between control and the 18 J/ cm^2 treated group, 0.68 ± 0.04, (p=ns) [Figure 2b]. In addition, there was evidence of reverse migration in 3J/ cm^2 as a few neutrophils migrated away from the wound. Static ratio or stasis which is defined as the retention of neutrophils in one area over time [14] was significantly lower in the treated 3 J/cm^2, 22 ± 0.01, (p=0.002) and 18 J/cm^2, 0.22 ± 0.01 (p=0.04) when compared to control, 0.31 ± 0.01, [Figure 2c]. Tortuosity or meandering index, which is defined as a ratio of cumulative distance to the shortest path traveled was increased by the 3 J/cm^2 treatment, 2.38 ± 0.34 when compared to control, 1.92 ± 0.34, (p=0.05) and 18J/ cm^2, 1.88 ± 0.34, (p=0.03) [Figure 2d].

Neutrophil recruitment was further analyzed according to three different time domains, post-injury: T1 (30-60 mpi), T2 (30-90 mpi), and T3 (30-120 mpi). For the three timepoints, the laser treated animals demonstrated a pattern of low neutrophil counts close to the wound (Quartile 1) and higher neutrophil counts furthest from the wound (Quartile 4), Figure 3 when compared to control animals, however this pattern did not reach statistical significance.

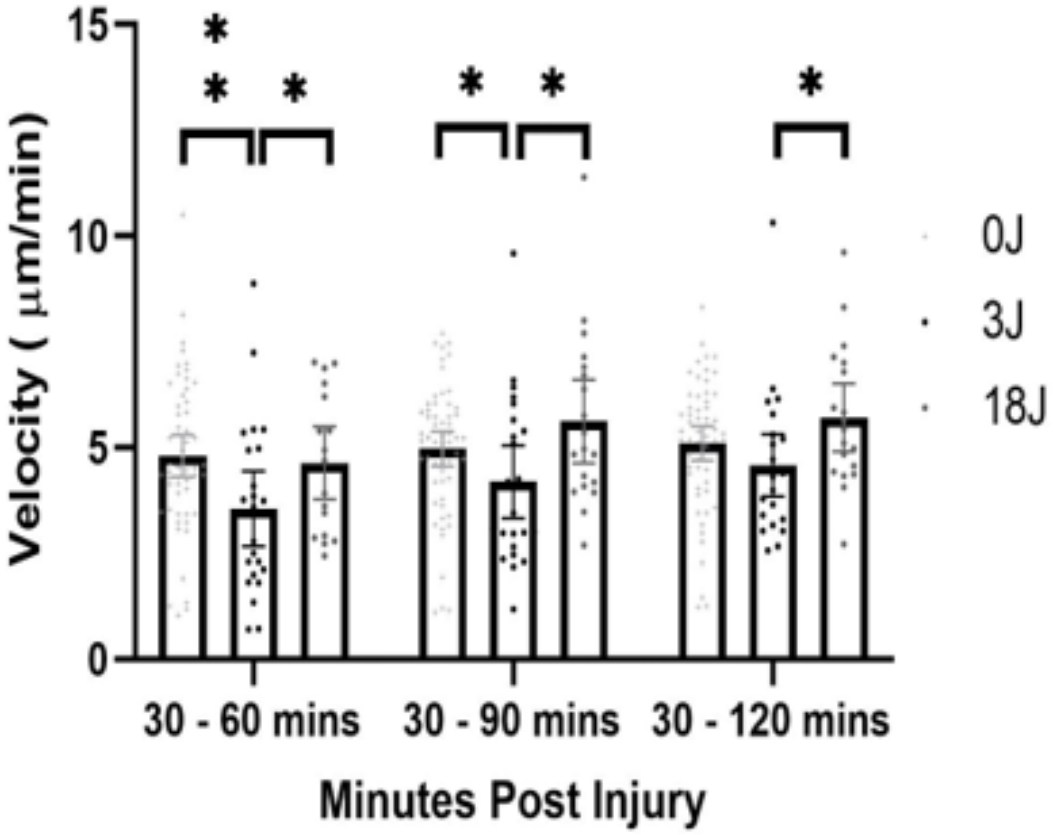

Figure 2A

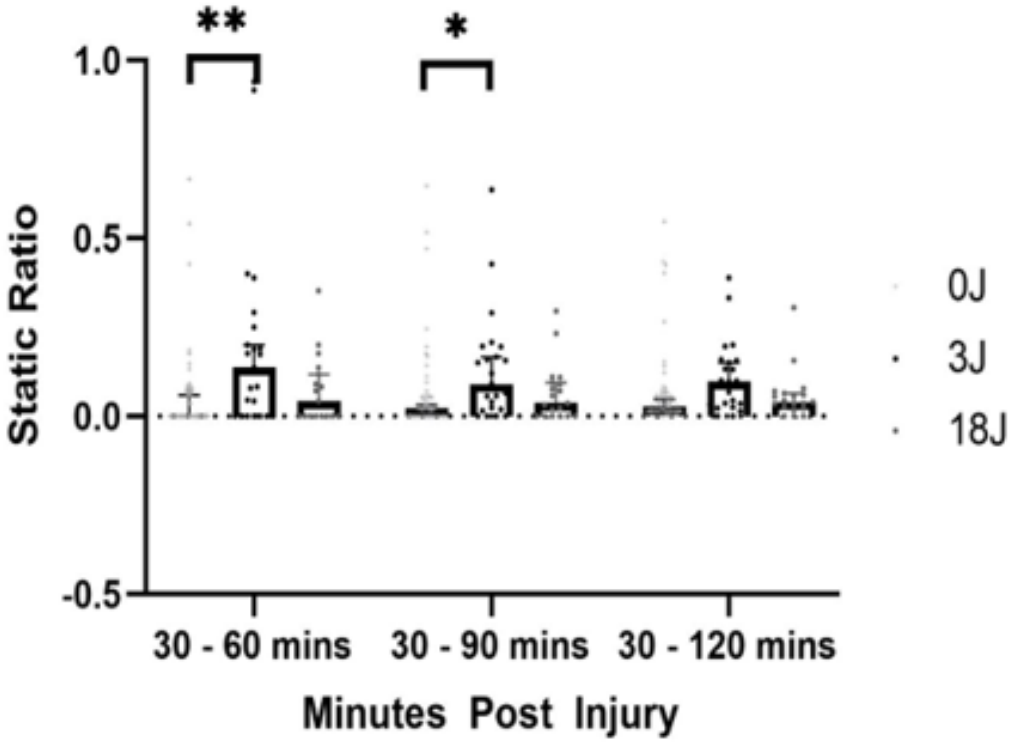

Figure 2B

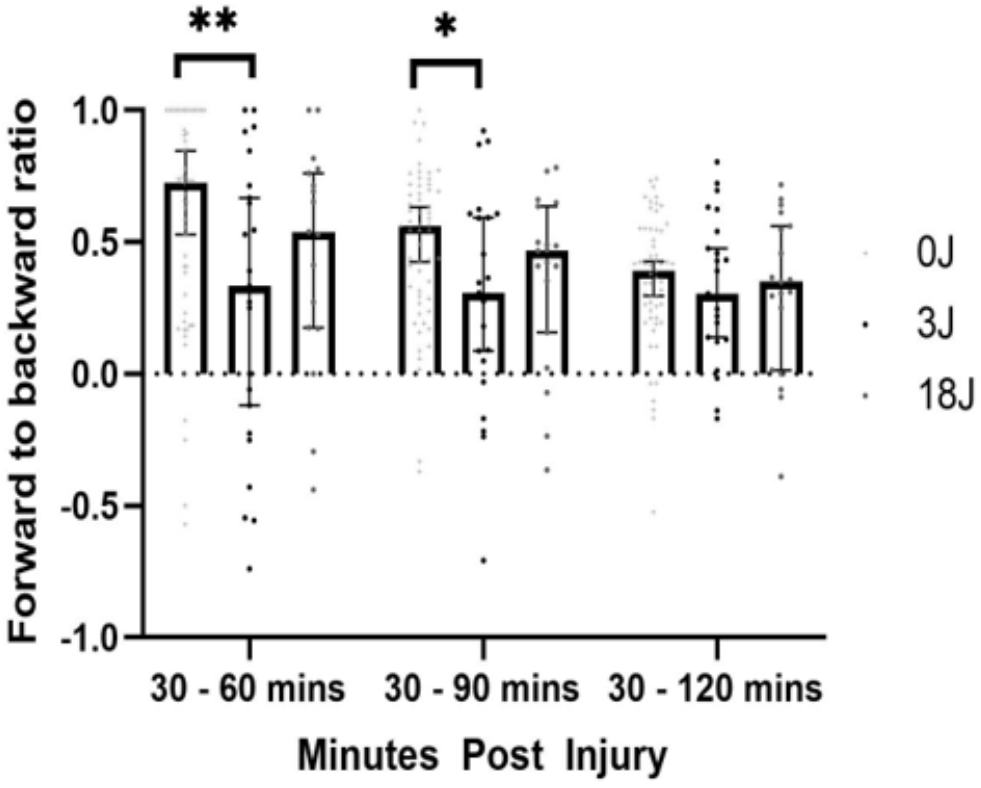

Figure 2C

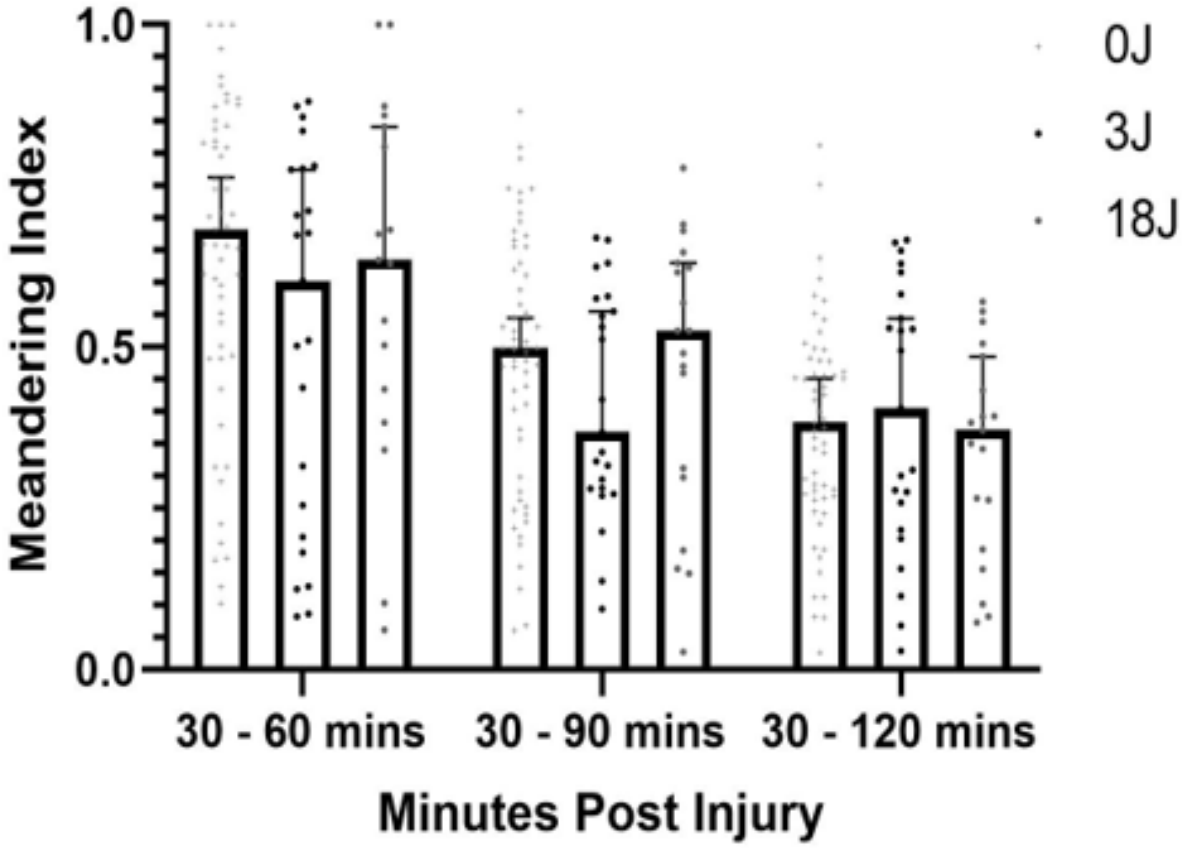

Figure 2D

Figure 2. *Display of total individual cell average of neutrophil migration metrics in spatial domain S2 of medians and 95% CI of dose groups (0J, n=51 cells; 3J, n=21 cells; 18J n=17 cells) showing velocity of cells traveling toward the wound, (A), Static Ratio, (B), demonstrating the retention of neutrophils in one area over time, Forward to backwards movement, (C), in reference to the wound center, and Meandering Index, (D). When comparing the 3J treated group to control or 18J treated group, significant differences were observed in velocity, static ratio, and forward direction, but not in meandering index (* = p<0.05 , ** =p <0.01). Statistical differences were computed with Student's t-tests (*p < 0.05), (**p < 0.01) in Graph*

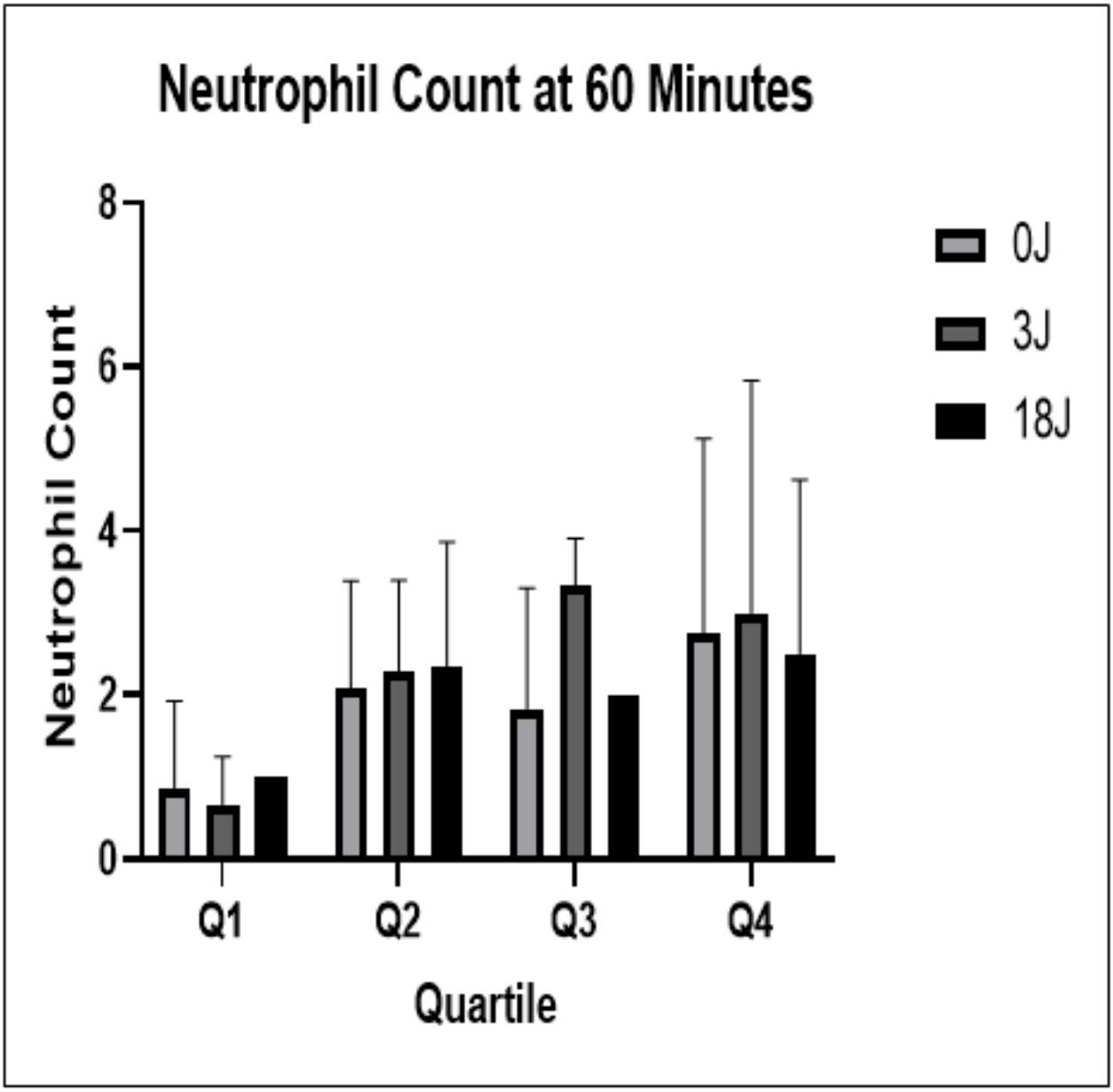
Neutrophil Count at 60 Minutes
Neutrophil Count
8
6
4
2
0
Q1
Q2
Q3
Q4
Quartile
0J
3J
18J

Figure 3A

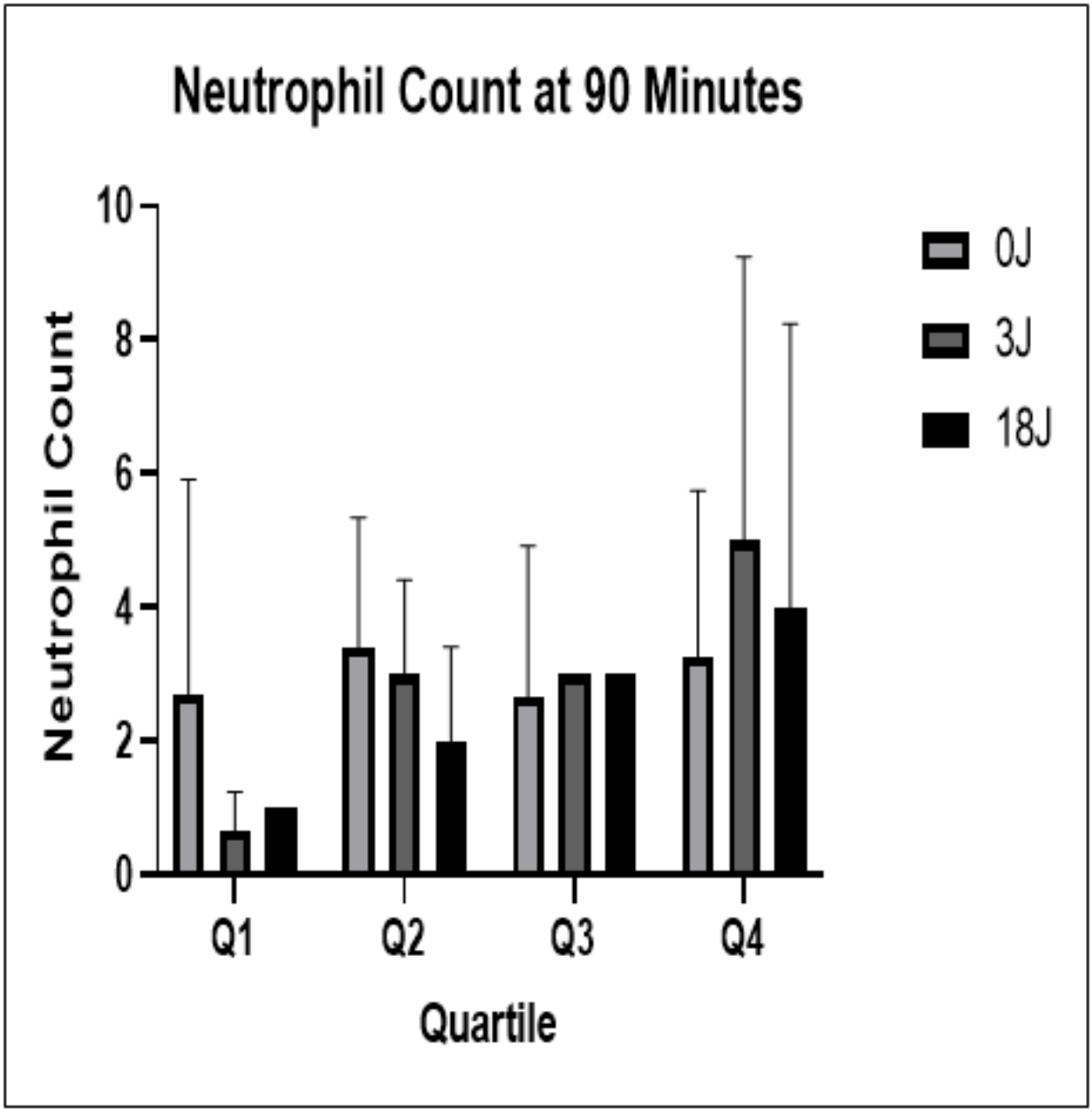
Neutrophil Count at 90 Minutes
Neutrophil Count
10
8
6
4
2
0
Q1
Q2
Q3
Q4
Quartile
0J
3J
18J

Figure 3B

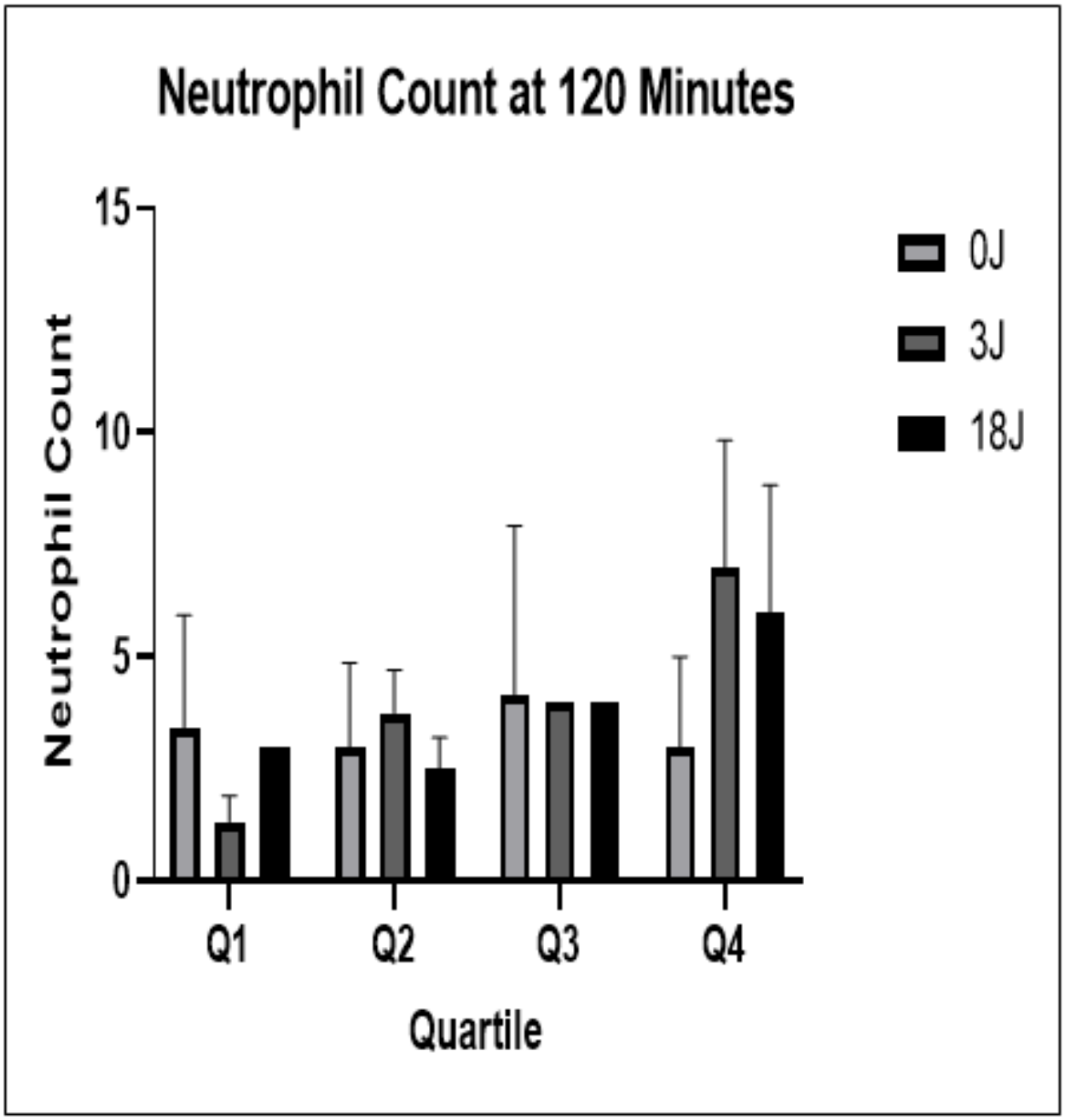

Figure 3C

Figure 3. *Neutrophil migration towards each quartile was measured with respect to three time intervals, 0-60 mpi (A), 60-90 mpi (B), and 90-120 mpi (C). Quartiles were assigned according to spaces characterized by their relationship to the wound: quartile one (s1), 150 micrometers encompassing the wound, from s0, center of wound defect, to the notochord, quartile two (s2),150 micrometers adjacent to s1, quartile three (s3), 150-300 micrometers from s1, quartile four(s4), 300-450 micrometers from s1.*

Discussion

The Zebrafish model was used as it facilitated quantitatively real time detection of a dose response in neutrophils. Real-time imaging generated over 1000 images which were processed to identify trends and significant differences over time and treatment groups. The lower dose fluence, 3 J/cm^2, was found to significantly decrease neutrophil migration speed to the wound, increased reverse migration, and promoted stasis in the area adjacent to the wound but not including the wound, spatial domain 2, when compared to the higher fluence or the naïve neutrophils. Also, unlike the naïve neutrophils which demonstrated a lot of forward and back movement to and from the wound, treated neutrophils seemed to have less movement like this and a greater reduction in the range of back and forth traveling. It is compelling that the same dose of 3J which led to macrophage increased velocity[12] actually slowed the neutrophils. Also, meandering was increased by the 3 J/cm^2 treatment, when compared to control and 18 J/cm^2 treated neutrophils.

These findings advance the notion that low fluence treatments reduce neutrophil inflammatory responses within the wound by their reduced presence within the wound. Low fluence treatments in concert with accelerated wound healing may be considered in the perspectives of a reduced neutrophil inflammatory response or an augmented reparative neutrophil response or their combination. Our data suggest enhanced energy leads to several changes in neutrophil kinetics which may be contributing to the reduction of their inflammatory influence within the wound. Reduced neutrophil-mediated collateral damage may work in concert with enhanced macrophage wound phagocytic activity to limit the pro-inflammatory wound healing stage and promote faster entry into the wound regeneration stage.

Additional potentially contributing mechanisms not defined within these studies could include the effect of fluence on neutrophil activation state, metabolic state, and maturation, all of which have been implicated in reduced inflammatory states or improved reparative states. Metabolic pathways used by neutrophils to power cellular activity have included glycolysis, the pentose phosphate pathway, oxidative phosphorylation with the mitochondria, the tricarboxylic acid cycle, and a fatty acid oxidation pathway[16]; neutrophils predominately use anaerobic glycolysis due to their function in microenvironments where oxygen and metabolic substrates are limited[17].

Glycolysis is less efficient than oxidative phosphorylation in ATP production but 100 times faster, enabling to neutrophils to rapidly function in high stakes, anaerobic conditions where immediate response times are critical[15]. Glycolysis is replenished by neutrophil gluconeogenesis/glycogenesis, enabling neutrophil bactericidal activity and survival[16]. Neutrophils also utilize the glucose-dependent Pentose Phosphate Pathway,

which is essential for neutrophil production of ROS[18]. The increased available energy supplied by the laser treatment may induce a metabolic switch away from these two pathways and skew neutrophils towards oxidation phosphorylation, leading to reduced inflammatory responses. In our previous study, laser treatment led to reduced levels of reactive oxygen species[12].

Oxidative phosphorylation by neutrophils has been described as instrumental in the drive from immature to mature neutrophils[20]. Mature neutrophils are not activated and do not produce significant amounts of inflammatory molecules. It is possible that the modest amount of extra energy delivered may have promoted the progression to a non-activated state in situ. Neutrophils have recently been implicated in tissue repair via multiple mechanisms: phagocytosis, release of growth and proangiogenic factors (ie, microvesicles containing AnxA1, microRNA-223), and the dampening of recruitment of additional inflammatory cells (ie NETs)[19]. Additional energy may enhance these functions and may also hasten their progression, possibly by the production of pro-resolving mediators such as resolvin and protectins which stop neutrophil migration by interfering with chemotactic signals.

Conclusion

In contrast to our previous research in macrophages, where low fluence treatment accelerated forward-directed migration into the wound, low fluence treatment significantly decreased neutrophil migration speed into the wound, promoted stasis outside the adjacent wound area, and increased reverse migration. Low fluence treatment groups exhibited more rapid wound closure. These results support the notion that low fluence therapies may decrease neutrophil inflammatory responses in the wound by reducing their presence within the wound. Minimized neutrophil-mediated collateral damage may work together with enhanced macrophage wound phagocytic activity to minimize the pro-inflammatory stage of wound healing and promote the wound regeneration stage.

Low fluence treatments together associated with accelerated wound healing may be considered in the perspectives of a reduced neutrophil inflammatory response or an augmented reparative neutrophil response or their combination. Our findings suggest that modest amounts of enhanced energy cause a number of modifications in neutrophil kinetics which may be contributing to a decrease in inflammation within the wound.

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Conflict of Interest Disclosure
The authors have nothing to disclose.

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